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Tumorigenesis and Hormone Therapy Resistance

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14. ABSTRACT The estrogen receptor (ER) plays a central role in the progression of breast cancer. Current endocrine therapy for ER+ve breast cancer involves Tamoxifen, and Aromatase inhibitors. De novo and/or acquired resistance to endocrine therapies however occurs frequently. Interestingly, most downstream events in these resistance signaling pathways converge upon modulation of cell cycle regulatory proteins, including upregulation of Cyclin E and A with activation of Cyclin Dependent Kinase 2 (CDK2). PELP1 (Proline, glutamic Acid and Leucine rich Protein) is a novel ER coregulator that has deregulated expression and localization in breast cancer. In the first year of grant support from DOD-BCMRP, we have found that PELP1 is a novel substrate of CDK2 and that its phosphorylation is important for estrogen mediated cell cycle progression using ZR75 model cells that harbors over-expressed dominant PELP1-CDK2 site mutant. We have also developed the first ever phospho-antibody against PELP1 to study its phosphorylation status and function in cell cycle progression and tumorigenesis. We also found that PELP1 can E2F transactivation function and future studies will include investigating the role of CDK2 mediated phosphorylation of PELP1 in epigenetic modulation of E2F and ER target genes, and thereby in PELP1 mediated tumorigenesis. In further we intend to characterize the CDK2-PELP1 axis in hormone therapy resistance.					
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I. INTRODUCTION

The estrogen receptor (ER) plays a central role in the progression of breast cancer. Current endocrine therapy for ER+ve breast cancer involves modulating ER-pathway using Tamoxifen, (1) and blocking peripheral estrogen (E2) synthesis by Aromatase inhibitors (2). Despite the positive effects, *de novo* and/or acquired resistance to endocrine therapies frequently occur. Although mechanisms for hormonal therapy resistance remains elusive, emerging data implicate growth factor signaling pathways and its cross talk with ER as major cause of resistance (3-5). *Interestingly, most downstream events in these resistance signaling pathways converge upon modulation of cell cycle regulatory proteins; the most conspicuous of which is the upregulation of Cyclin E and A, along with activation of Cyclin Dependent Kinase 2 (CDK2)* ((6-10). Activation of CDK2 in these resistant tumors is reported to be brought about by downregulation of p27-CDK inhibitors (11-13) or by functional ablation of Retinoblastoma (pRb) and further upregulation of Cyclin E (14, 15). ER signaling complexes are known to recruit various co-regulatory proteins and recent evidences suggest that deregulated expression, localization and activity of ER coregulators also plays vital role in endocrine resistance (16); examples include SRC-1 (17), SRC3 (AIB1) (18-20), NCOR1 (21) and PELP1 (22). ***The major focus of this proposal will be on PELP1*** (Proline, glutamic Acid and Leucine rich Protein), a novel ER coregulator (23, 24) normally expressed in mammary gland but shows deregulated expression and localization in breast cancer (25). Emerging studies implicate PELP1 as a proto-oncogene (26) and its deregulation might play a role in hormone therapy resistance (27). My preliminary results suggest that PELP1 is a novel substrate of CDK2. Based on these rationales, I hypothesize that ***a) phosphorylation of PELP1 by CDK2 confers growth advantage to breast epithelial cells via upregulating ER/ E2F target genes and histone biosynthesis, thus contributing to its oncogenic potential; b) deregulation of CDK2-PELP1 signaling axis constitutes a novel signaling pathway towards acquired hormonal therapy resistance.***

II. BODY

The scope of this proposal is to undertake the following three tasks outlined in the approved statement of work:

Task 1. To determine the biological significance of CDK2 mediated phosphorylation of PELP1

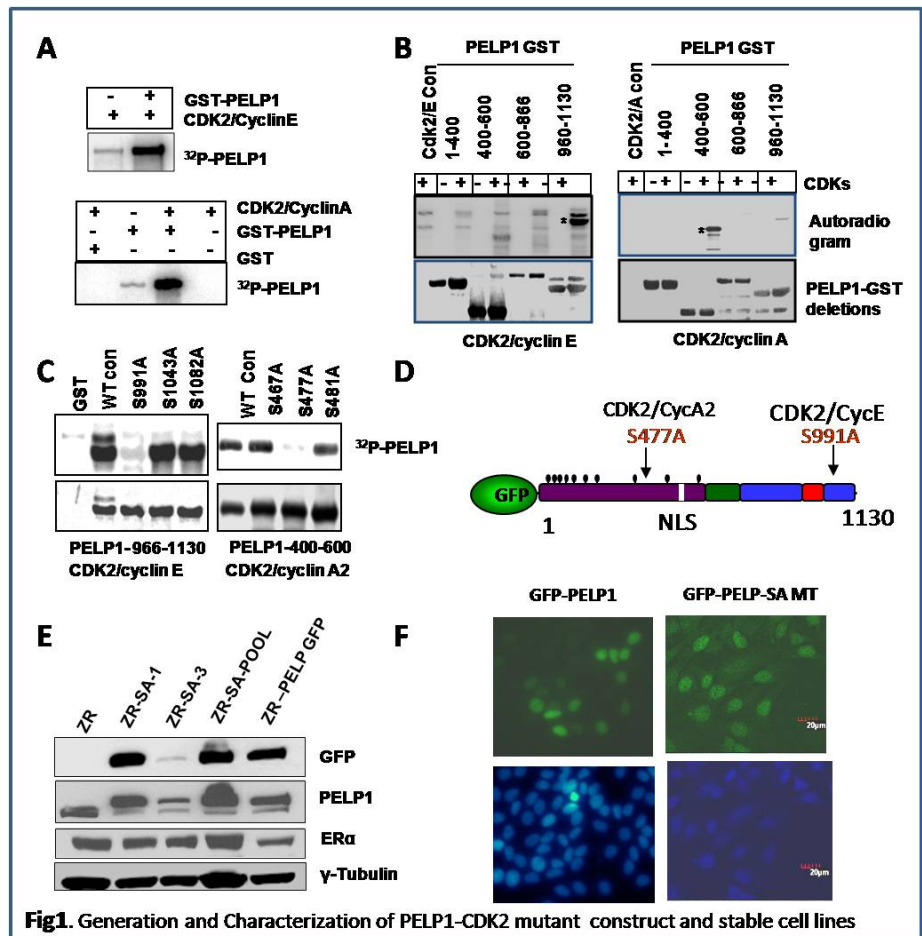
Task2. To understand biological significance of CDK2 mediated phosphorylation of PELP1 on histone biosynthesis and PELP1's interactome

Task3. To investigate the therapeutic potential of CDK-PELP1 axis in tumorigenesis and hormonal therapy resistance

TASK I:

a) Generation of PELP1-CDK mutant plasmid constructs and model cells:

We have found that CDK2/CycE and CDK2/CycA2 both phosphorylate PELP1 (Fig1A) and utilizing various deletion mutants (Fig 1B) and site directed mutagenesis of putative CDK2 phosphorylation sites on PELP1, we have identified Ser 991 and Ser 477 as the CDK2 phosphorylation sites (Fig 1C). To further study the *in vivo* significance of CDK phosphorylation of PELP1, an N-terminal GFP-tagged PELP1 mutant that lacked these two CDK sites were generated using site directed mutagenesis (Ser to Ala mutant) (Fig 1D). ZR75 cells stably expressing PELP1-WT and PELP1-MT (single and pooled clones) were generated. ZR75 cells were

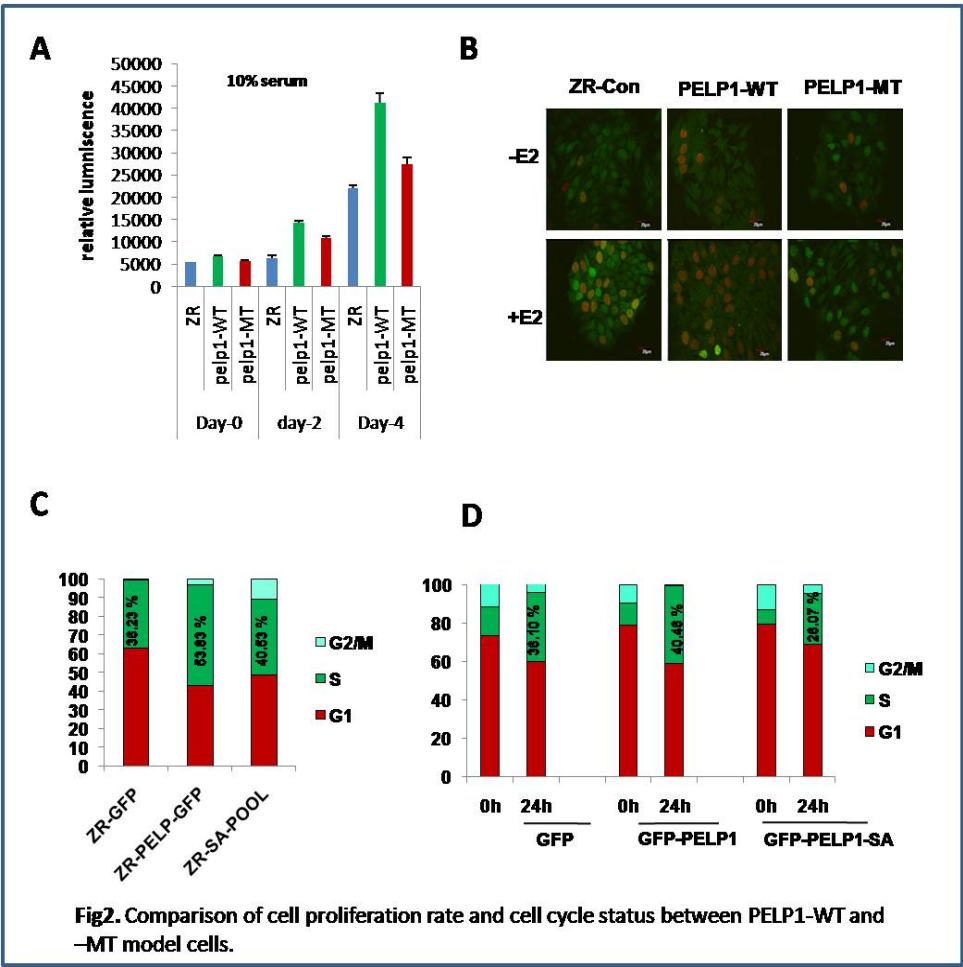


chosen based on our earlier findings that ZR cells express relatively low levels of endogenous PELP1,

and thus were more suitable to study the functions of exogenously expressed PELP1 than other ER-positive cells such as MCF7. In general, these stable clones express 3- to 4-fold more PELP1-MT than endogenous PELP1, and mutant expression is equivalent to GFP-tagged PELP1-WT clone and migrated to the expected size on SDS-PAGE when detected using the GFP antibody (Fig. 1E). Both PELP1-WT and PELP1-MT localized to the nuclear compartment when detected employing immunofluorescence (Fig.1F). Phosphorylation mimetic constructs (Ser to Glu mutant) has been constructed and confirmed by sequencing.

b) Effect of CDK2 phosphorylation site mutations in PELP1 on E2 mediated cell cycle progression:

To test if the CDK2 phosphorylation contributed to PELP1's ability to drive cell cycle progression, we compared the cell proliferation rate between PELP1-WT and PELP1-MT expressing model cell lines using a Cell Titer-Glo Assay. Asynchronized cells (ZR-75 controls, PELP1-WT, PELP1-MT) were plated equally in a 96 well clear plate and after 48 and 96 h, rate of proliferation was assayed following manufacturer's instructions. As expected from the previous studies, PELP1-WT expression increased cell proliferation compared ZR75 control cells. CDK2 site mutants clearly lagged behind the PELP1-WT cells (Fig 2A).



To assess how these mutant cells respond to estrogen (E2) treatment as compared to PELP1-WT cells, we employed BrdU labeling assay. ZR75 stably expressing GFP or GFP tagged PELP1-WT or GFP tagged PELP1-MT were synchronized to G₀ phase by serum starvation, cells were labeled with BrdU and released into cell cycle by addition of 10% FBS containing serum for 16 h. Results show that PELP1-WT cells acquire more BrdU into DNA depicting increased cells in S phase of cell cycle while PELP1-MT clearly show less cells with BrdU labeling (Fig. 2B). We further analyzed the cell cycle progression of the PELP1-WT and -MT clones by flow cytometry and found that PELP1-WT expression contributed to increased G1-S progression, while mutation of the CDK sites in PELP1 diminished its ability to a great extent (Fig. 2C). Similarly, mutation of the CDK sites in PELP1 decreased the number of cells entering S phase upon E2 stimulation (Fig. 2D). Overall, these results suggest that CDK2 mediated phosphorylation is important for PELP1 mediated cell cycle progression.

c) Generation and characterization of phosphorylation-state-specific-antibodies for PELP1:

To further characterize the *in vivo* relevance of the identified sites, we made an attempt to generate rabbit polyclonal phospho-specific antibodies against each of phospho-S991 and phospho-S477 sites using commercial facility from

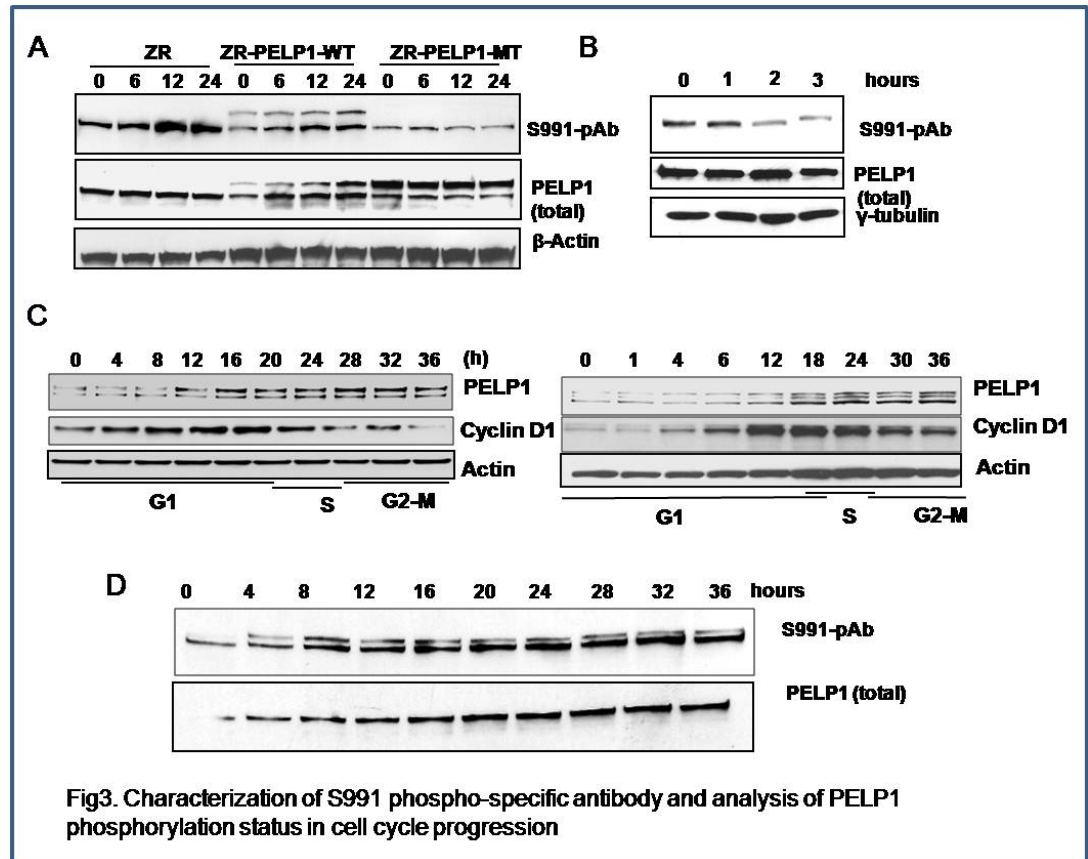
Openbiosystems (Fischer Sci). The peptide sequence used for generating phosphoantibody against S477 and S991 were SPPADALKLR(pS)PRGSPDGS LQ and TLPPALPPPE(pS)PPKVQPEPEP respectively. We were successful only in obtaining S991-phospho-antibody, while failed to generate S477-phospho-antibody; probably because of the poor antigenicity of the S477 peptide. The PELP1 S991 antibody was affinity purified using positive absorption with phospho-peptide followed by negative adsorption with unmodified peptide. The purified antibody

Fraction	Peptide #	Volume (mL)	Conc. (mg/mL)	Total (mg)	Titer
Phos Specific antibody	S991-Phospho-peptide	13	0.224	2.912	20ng
Phos Specific antibody	S991 non-phospho-peptide	-	-	-	40ng

Table1. Purified antibody is titered by indirect ELISA against the phospho- and non-phospho-peptide bound to the solid-phase to measure the reactivity of the antibodies after elution (the eluent).The eluent titer indicates the minimum concentration at which purified antibody can effectively detect the antigen

titer was quantitated by indirect-ELISA (Table 1). The antibody efficiently recognized phosphorylated

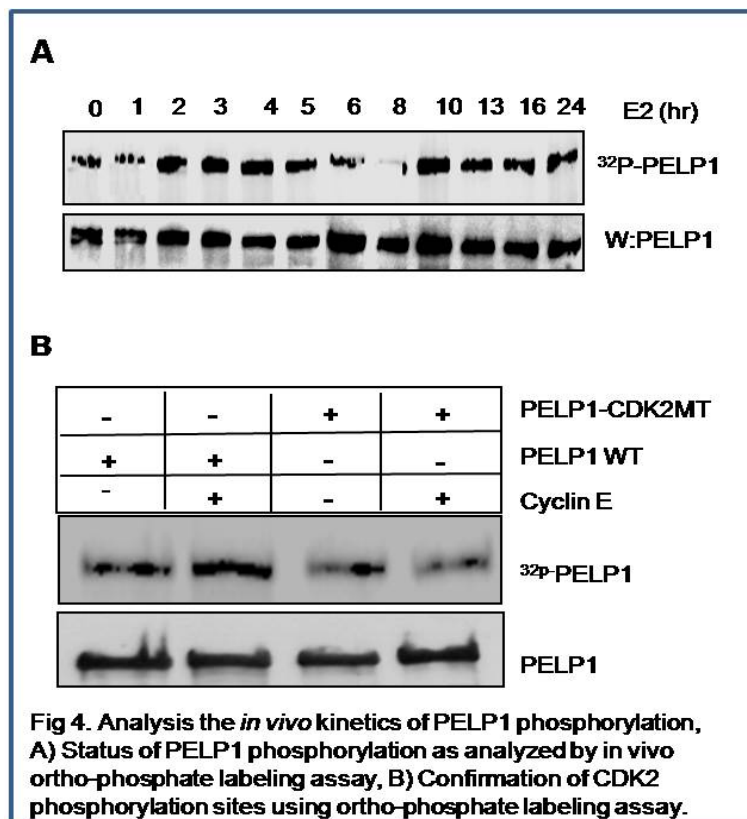
endogenous and GFP-tagged PELP1, but was unable to detect the Ser991 to Ala PELP1 mutant (Fig 3A). We then analyzed the *in vivo* PELP1 phosphorylation status in MCF7 using double thymidine block and release and then by Western blotting using phospho-S991 PELP1 antibody. We found that PELP1 Ser991 phosphorylation accumulated at



the G1-S boundary and gradually decreased thereafter as the cells progressed into other phases of the cell cycle (Fig. 3B). Similarly, normal transformed IMR-90 and NIH3T3 were synchronized to G1 phase by serum starvation and released into the cell cycle by addition of 10% serum and the expression of PELP1 was analyzed on a 4-12% Bis-Tris gradient gel. We found that human fibroblast cells exhibited two different mobility shifts in PELP1; a slow-migrating form and a fast-migrating form (Fig. 3C, left panel) while in murine fibroblast cells there were two slower moving forms during various phases of cell cycle (Fig.3C, right panel). To examine whether the PELP1 Ser991 antibody recognized the shifted bands seen in normal IMR-90 cells, we synchronized IMR-90 cells to G1 phase and released them into the cell cycle. Analysis of cell lysates on a 4-12% gradient gel revealed that the phospho PELP1 antibody recognized both forms (Fig 3D). Recognition of the two forms by PELP1 phospho-specific antibody suggests that PELP1 gets phosphorylated at multiple residues upon cell cycle progression (hypo and hyper-phosphorylated states) similar to the other cell cycle regulatory protein pRb. Overall these results demonstrate that interphase CDKs can phosphorylate PELP1 *in vivo*.

c) Studying the *in vivo* kinetics of PELP1 phosphorylation:

To validate whether PELP1 is phosphorylated *in vivo*, we synchronized MCF7 cells to G₀/G₁ by serum starvation, labeled the cells with ³²P-orthophosphate and treated the cells with E2 for different periods of time. We then performed immunoprecipitation of PELP1 using endogenous PELP1 antibody and found that PELP1 exists as a phospho-protein with two peaks of phosphorylation (early 2-5 h and later 10-13 h) after E2 stimulation (Fig. 4A). To confirm the CDK2 phosphorylation sites in PELP1, 293T cells were co-transfected with either PELP1-WT or PELP1-MT construct with or without Cyclin E followed by ortho-phosphate labeling and Immunoprecipitation using anti-GFP antibody. Autoradiograph confirmed that the CDK2 site mutations in the context of full-length PELP1 significantly reduce CDK2/CycE- mediated PELP1 phosphorylation (Fig 4B).



d) Effect of CDK2 phosphorylation on PELP1 mediated E2F transactivation function: Since PELP1 is a pRb binding protein and CDKs phosphorylate PELP1, we investigated whether CDKs phosphorylation of PELP1 aids in E2F functions. First, we compared the expression of genes involved in cell cycle progression between MCF7 and MCF7-PELP1-shRNA stable cells using a focused microarray approach. Commercially available Oligo GEArray® Human Cell Cycle Microarray (SABiosciences, Frederick, MD) that contains 112 genes involved in cell cycle regulation was used and target genes whose expression was differentially regulated (at least 2 fold difference) upon PELP1 depletion were identified. Down regulation of PELP1 substantially reduced the expression of a number of cell cycle genes including Cyclin D1, pRb, cyclin B2 and CDC25C (Fig 5A). We then examined whether PELP1 enhances E2F-mediated gene activation and whether CDK phosphorylation affects PELP1 activation of E2F functions using E2F luciferase reporter. PELP1 knock down substantially

reduced the E2F reporter gene activity (Fig 5B, left panel). The cells with PELP1-WT overexpression had greater E2F luciferase reporter activity, while PELP1-MT that lacked CDK phosphorylation sites failed to enhance the E2F reporter activity (Fig.5B, right panel). Real-Time PCR also demonstrated that many of the E2F target genes are downregulated upon PELP1-siRNA treatment in MCF7 cells (Fig 5C). Chromatin immunoprecipitation demonstrated that PELP1 is recruited to the promoters of the E2F target gene promoters Cyclin A and Cyclin E that contain E2F binding sites (Fig. 5D). However mutation of CDK phosphorylation sites in PELP1 did not affected its recruitment over E2F target genes (5E). The effect of PELP1 CDK2 mutants on ERE luciferase reporter activity will be studied in future.

Fig 4. Significance of CDK2 mediated phosphorylation of PELP1. A) Profiling cell cycle gene expression in MCF7 and PELP1 –shRNA MCF7 cells, B) Effect of CDK2-mutations on 4XE2F luciferase reporter assay, C) RT-PCR analysis of E2F target genes, D) ChIP assay showing recruitment of PELP1 over classical E2F target genes, E) Effect of CDK2 mutations on PELP1 recruitment over E2F target genes during cell cycle progression.

III. KEY RESEARCH AND TRAINING ACCOMPLISHMENTS

A. Key Research Accomplishments:

- Establishment of breast model cells model cells with functional and CDK2-defective PELP1 signaling axis
- Demonstration that endogenous PELP1 phosphorylation is needed for optimal estrogen mediated G1-S cell cycle progression

- Demonstration of the significance of PELP1 in E2F signaling.
- Generation of a novel phospho-specific PELP1 antibody and characterization.

B. Key Training Accomplishments:

- **Journal Club Attendance and Presentations:** I have attended “Hormones and Cancer Journal Club” organized bi-weekly at Dept of OB-GYN and presented critical papers from top journals three times during last one year.
- **Department Seminar Series and Lab meetings:** I have attended all the mandatory Seminars and invited speech organized at Dept of Molecular Medicine. In addition to that, I have participated in all lab meetings organized in Dr Vadlamudi lab on a weekly basis.
- **Oral Presentations:** Have presented my data during Annual Departmental Retreat, held on Sept 2009 (Dept of Molecular Medicine).
- **Teaching and Supervising:** Have completed all my teaching assistant duties (2008-09) at Dept of Molecular Medicine. I also had the opportunity to supervise a high school student on her summer research project (2009).
- **Conferences/Symposiums:** I have attended the SABCS (San Antonio Breast Cancer) symposium. In addition, I have also presented a poster at Annual CTRC-San Antonio Cancer Institute Retreat (2009).
- **Honors and Awards:** I have been awarded the “Novartis Oncology Basic Science Scholar Award” for my poster presented at SABC symposium (2008). I also received ‘Second Prize’ for the oral presentation at Annual Dept Retreat held at Dept of Molecular Medicine (2009).

IV. REPORTABLE OUTCOMES

A. Publications:

1. **Nair BC** and Vadlamudi RK. Regulation of hormonal therapy Resistance by cell cycle machinery. *Gene Therapy and Molecular Biology*. 12:395-404, 2008.
2. **Nair BC** and Vadlamudi RK. ZD6474 coerces breast cancer for an apoptotic journey. *Cancer Biology and Therapy*. 9:8, 1-3; 2010

B. Abstracts:

1. **Chandrasekharan NB**, Nair SS, Chakravarty D, Yew RP, Tekmal RR, **Vadlamudi RK**.. Modulation of hormone therapy resistance by CDK2-PELP1 axis 2009 Jan. (Cancer Research; vol. 69, no. 211S **Abstract #3022**).

V. CONCLUSIONS: E2 is known to promote key cell cycle events like activation of CDK and hyperphosphorylation of pRB in ER-positive breast epithelial cells, leading to increased rate of G1/S phase transition. In the first year of grant support from DOD-BCMRP, we have found that PELP1 is a novel substrate of CDK2 and that its phosphorylation is important for estrogen mediated cell cycle progression using ZR75 model cells that harbors overexpressed dominant PELP1-CDK2 site mutant. We have also developed the first ever phospho-antibody against PELP1 to study its function in cell cycle progression and tumorigenesis. Future studies will include investigating the role of CDK2 mediated phosphorylation of PELP1 in epigenetic modulation of E2F and ER target genes, and thereby in PELP1 mediated tumorigenesis and also characterizing the CDK2-PELP1 axis in hormone therapy resistance. We will also explore whether CDK2 inhibitor roscovitine reverses therapy resistance. These studies will be done as a part of 2nd and 3rd Year of continued funding from Department of Defense.

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VI. APPENDICES

- 1. Nair BC** and Vadlamudi RK. Regulation of hormonal therapy Resistance by cell cycle machinery. Gene Therapy and Molecular Biology. 12:395-404, 2008.

Regulation of hormonal therapy resistance by cell cycle machinery

Review Article

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Key words: Cell Cycle, CDKs, Estrogen, Estrogen Receptor, Co-regulators, Breast cancer, Therapy resistance, Antiestrogens

Abbreviations: cyclin-dependent kinase, (CDK); epidermal growth factor, (EGF); estrogen receptor, (ER); mitogen-activated protein kinase, (MAPK); modulator of nongenomic actions of the ER, (MNAR); nuclear receptor, (NR); phosphatidylinositol-3 kinase, (PI3K); proline-, glutamic acid-, and leucine-rich protein, (PELP); protein kinase A, (PKA); retinoblastoma protein, (pRb)

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Summary

Estrogen Receptor (ER) plays a central role in the development and progression of breast cancer. Hormonal therapy substantially improves disease-free survival of ER+ve breast tumors, however acquired resistance to endocrine therapies frequently occur. Emerging data implicate growth factor signaling pathways and their cross talk with ER as major cause of resistance. Both these pathways have been recently shown to use cell cycle machinery as downstream effectors in mediating therapy resistance. Several studies have demonstrated deregulation of cell cycle regulators and their cross talk with ER in therapy resistant tumors. The objective of this article is to review the underlying mechanisms by which tumor cells use cell cycle machinery to override hormonal therapy and to explore cell cycle machinery components as novel therapy targets for overcoming hormonal therapy resistance.

I. Introduction

Steroidal hormone estradiol (E2) and Estrogen Receptor (ER) plays a central role in the development and progression of breast cancer and 70-80% of breast tumors are ER positive at the time of presentation (McGuire and Clark, 1992). ER positive tumors respond well with therapeutic agents targeting ER functions (Ariazi et al, 2006). Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator (SERM), has been shown to improve relapse-free and overall survival (Lewis-Wambi and Jordan, 2005). More recently, aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women (Leary and Dowsett, 2006). Despite the success of antiestrogens, de novo and/or acquired resistance to endocrine therapies frequently occur. Approximately 30% of these patients acquire resistance to endocrine therapy in later stages and is a significant problem in the treatment regime (Riggins et al, 2007). Although mechanisms for hormonal therapy resistance remain elusive, emerging data implicate growth

factor signaling pathways and its cross talk with ER as a major cause of resistance (Shou et al, 2004). Both these pathways have been recently shown to use cell cycle machinery as downstream effectors in mediating therapy resistance (Shou et al, 2004; Perez-Tenorio et al, 2006; Ru et al, 2006). The prime focus of this review is to recapitulate the literature elucidating the role of cell cycle machinery as downstream effectors of various pathways leading to hormone therapy resistance.

II. Estrogen receptors and coregulators

The human estrogen receptor (ER) is a key transcriptional regulator in breast cancer biology (Green and Carroll, 2007; Heldring et al, 2007). The biological effects of estrogen is mediated by its binding to the structurally and functionally distinct ERs (ER α and ER β) (Warner et al, 1999). ER α is the major ER in the mammary epithelium and this has been further shown by ER α (Esr1) knockout mice, which display grossly impaired ductal epithelial cell proliferation and branching

(Lubahn et al, 1993; Bocchinfuso and Korach, 1997). ERs comprises an N-terminal activation function 1 (AF1) domain, a DNA-binding domain, and a C-terminal ligand binding region that contains an activation function 2 (AF2) domain (Kumar et al, 1987). The AF-2 of the ER is located in the ligand binding domain, while the N-terminal AF-1 functions in a ligand-independent manner. AF-1 and AF-2 exhibit cell type and promoter context specificity (Berry et al, 1990). Upon binding of E2 to ER, the ligand-activated ER translocates to the nucleus, binds to the responsive element in the target gene promoters, and stimulates gene transcription (genomic/nuclear signaling) (McKenna et al, 1999; McDonnell and Norris, 2002). In addition to its well-studied nuclear functions, ER also participates in non-genomic signaling events in the cytoplasm and membrane. Such signaling has been linked to rapid responses to E2 which generally involves the stimulation of the Src kinase, MAPK, and AKT (Pedram et al, 2002).

Transcriptional activity of ERs is regulated not only by hormones but also by several coregulatory proteins called coactivators and corepressors (McKenna et al, 1999). The transcription functions of ER are shown to be influenced by several coactivators, including SRC1, GRIP1, AIB1, PELP1 and corepressors including nuclear receptor corepressor (NCoR), silencing mediator for retinoic and thyroid receptor (SMRT) and MTA1 (Tsai and O'Malley, 1994; Barnes et al, 2004). Coactivators preferentially associate with ligand bound ER while corepressors have been shown to preferentially associate with antagonist occupied ERs (Jepsen and Rosenfeld, 2002). Evidence suggests that multi-protein complexes containing coactivators, ERs, and transcriptional regulators assemble in response to hormone binding and activate transcription (McKenna et al, 1999). Accumulating evidence suggests that ER-coregulators play an essential role in hormonal responsiveness and cancer progression (Bocchinfuso and Korach, 1997; McKenna et al, 1999).

III. Estrogen and cell cycle progression

It is well accepted that estrogen induces mitogenesis by recruiting non-cycling cells into the cell cycle and by increasing the rate of progression from G₁ to S phase. However, the molecular mechanism by which E2-ER signaling controls cell proliferation is not completely understood. Induction of the early-response genes (such as c-myc and c-fos) is proposed as one mechanism of this process (Prall et al, 1998a; Lamb et al, 2000), whereas regulation of Cyclin Dependent Kinase (CDK2 and CDK4) activities was proposed as another (Neuman et al, 1997; Prall et al, 1997; Foster et al, 2001). Each phases of cell cycle (G₁, S, G₂ and M) is strictly under the control of different Cyclins and CDKs. CDK4 and CDK2 enhance G₁-S transition in the cell cycle and for tumorigenesis, indicating that phosphorylation of downstream effector proteins by CDKs is vital for cell proliferation. Previous studies have shown that Cyclin D1-CDK4 and Cyclin E-CDK2 are major regulators of G₁/S transition, while Cyclin A-CDK2 controls S-phase and Cyclin B1-CDK1

controls transition through M-phase. The kinases are traditionally known to phosphorylate many key downstream substrates, most notably retinoblastoma and exhibit strict and elegant control of cell cycle progression. In addition, Cyclin D1 was identified as a target of E₂ action, and estrogen treatment was shown to up-regulate Cyclin D1 levels (Altucci et al, 1996). Up-regulation of Cyclin D1 by ER signaling is accompanied by an increased proliferative response in breast cancer cells. E2 is shown to induce Cdc25A, a tyrosine phosphatase that controls G₁-S transition in cell cycle by regulating the dephosphorylation of Cyclin-dependent kinase complexes (Ru et al, 2006). Collectively, these findings suggest that Estrogen induces proliferation of ER-positive breast epithelial cells by stimulating G₁/S transition, which is associated with increased cyclin D1 expression and activation of CDKs (Foster et al, 2001). Since CDK4 and CDK2 are key players for G₁-S transition in the cell cycle and for tumorigenesis, ER crosstalk with CDKs will have implications in therapy resistance.

IV. ER coregulators and cell cycle progression

Evolving evidence suggests that many of the ER coregulators play a vital role in cell cycle progression. Emerging evidence suggest that oncogenic ER-coregulatory proteins such as AIB1, PELP1 modulate Cyclin D1 expression and function, thus may enhance tumorigenesis and therapy resistance. We have summarized below some of the ER coregulators that are shown to play a role in E2-ER mediated cell cycle progression.

A. AIB1

ER coregulator SRC3/AIB1 is shown to regulate cell cycle machinery in numerous ways. AIB1 is shown to enhance E₂-dependent induction of Cyclin D1, suggesting a role for ER coregulators in modulating Cyclin D1 expression (Planas-Silva et al, 2001). AIB1 is also shown to interact with E2F directly and modulate its transactivation function and is required for E2F1-mediated gene expression (Louie et al, 2004). Recent evidence also suggests that AIB1 has oncogenic potential and the transformation ability of AIB1 has been ascribed to its ability to control the expression of genes important for initiating DNA replication like cdc6, MCM7, Cyclin E, and CDK2 (Louie et al, 2006). E2F regulates AIB1 expression by cooperating with the transcription factor specificity protein 1 (Sp1) without direct interaction with E2F consensus sites, suggesting a positive feedback regulatory loop comprising of E2F and AIB1 (Mussi et al, 2006).

B. Ciz1

Ciz1, a p21(Cip1/Waf1)-interacting zinc finger protein is shown to function as an ER co-regulator and Ciz1 over-expression confers estrogen hypersensitivity and promotes the growth rate, anchorage independency, and tumorigenic properties of breast cancer cells. These effects on cell cycle progression is shown to be ER

dependent through upregulation of Cyclin D1 expression (Den et al, 2006). However, a direct role of Ciz1 in DNA replication process in S phase has also been suggested. Ciz1 co-localizes with PCNA during S phase while depletion of Ciz1 restrains cell proliferation by inhibiting entry to S phase (Coverley et al, 2005).

C. CARM1/PRMT4

CARM1 is a methyltransferase that associate with ER coregulators and regulate transcription by histone H3 methylation and is essential for estrogen induced cell cycle progression (Chen et al, 1999). SiRNA mediated depletion of CARM1 in ER positive MCF7 and T47D cells reduced E2 mediated cell cycle progression (Frietze et al, 2008). Recent evidence also suggest that CARM1 regulate not only E2 mediated E2F expression but also expression of E2F target genes. The recruitment of CARM1 to E2F target genes and associated increase in H3R17 dimethylation during transcriptional activation has been shown to be dependent on another ER coactivator AIB1 (Frietze et al, 2008). In a recent study, expression of Cyclin E gene has been shown to correlate with recruitment of CARM1 on its promoter and associated increase in H3-R26 and H3-R17 methylation at its promoter (El et al, 2006). Consistent with the role of CARM1 in regulating cell cycle genes, CARM1 knockout mice show small embryos and perinatal lethality (Yadav et al, 2003).

D. PELP1/MNAR

PELP1 is another ER coregulator that is shown to play a role in E2-mediated G1/S-phase progression (Balasenthil and Vadlamudi, 2003). PELP1 is a pRb-interacting protein and PELP1 deregulation promotes cyclin D1 expression. Breast cancer model cells, which overexpressed PELP1 showed persistent hyperphosphorylation of the pRb protein in an E2 dependent manner accompanied with increase in proliferation rate (Balasenthil and Vadlamudi, 2003). Recent studies suggested that PELP1 is a phospho-protein and its phosphorylation changes during cell cycle progression. PELP1 interacts with G1/S phase CDKs (both CDK4 and CDK2), and is a novel substrate to both of these enzymes (Chandrasekharan Nair et al, 2008a). Furthermore, increased PELP1 expression in a mammary gland during pregnancy, when the rate of cell proliferation is high, supports a physiological role for PELP1 in E2-mediated cell cycle progression in mammary glands (Vadlamudi et al, 2001). PELP1 is also known to interact with key proteins like Src, PI3K, four and a half LIM only protein2 to mediate E2 dependent non-genomic functions of ER. Mitogenic stimulus promotes PELP1 interaction with growth factor signaling component, epidermal growth factor (EGFR), HER2, STAT3, and hepatocyte growth factor regulated tyrosine kinase substrate (HRS) (Vadlamudi and Kumar, 2007). PELP1 has highest tissue expression in brain, testes, ovary and uterus (Khan et al, 2005; Vadlamudi and Kumar, 2007) and studies from rodent biology suggest that PELP1 is developmentally regulated and expressed at classical steroid target sites in brain like hippocampus, cortex, hypothalamus, amygdale

and septum (Khan et al, 2005). Collectively, these emerging findings suggest that PELP1 plays a key role in relaying mitogenic signals, both in cytoplasm and nucleus and therefore is an attractive therapeutic target. The fact that siRNA mediated knockdown of PELP1 reduces cell proliferation in MCF-7 breast cancer cells strongly suggest that blocking PELP1 functions will undeniably benefit cancer therapeutic regime (Chandrasekharan Nair et al, 2008).

V. Modulation of cell cycle progression by anti-estrogens

Estrogens and anti-estrogens both are shown to exert their functions in G1 phase, where they regulate Cyclin D1 and Cyclin E expression and hence modulate the kinase function of CDK4 and CDK2, respectively. Inhibition of CDK kinase function leads to accumulation of hypophosphorylated retinoblastoma and resulting in cell cycle arrest. In short, the consensus is that estrogen accelerates the G1 phase passage while antiestrogens inhibit cell cycle progression by affecting these key cell cycle proteins. On the contrary, a recent transcriptional profiling presented a rather intriguing result regarding functioning of tamoxifen at the molecular level. Tamoxifen and estrogen both positively regulated a large set of cell cycle genes like c-myc, myb, fos, cdc25a, Cyclin E, Cyclin A2, and stk15 while the differential effect was on only few cell cycle genes, most notably on Cyclin D1 (Hodges et al, 2003). Interestingly, only Tamoxifen but not Raloxifene induced these key cell cycle regulators (Hodges et al, 2003).

Emerging evidence suggest that CDK inhibitors are also regulated by antiestrogens in mediating growth arrest. Tamoxifen treated breast cancer cell lines show a reduction in Cyclin D, increase p27 and simultaneous increase in Cyclin E-CDK2 bound p27 (Chu et al, 2005). In the same study, combination treatment of Tamoxifen along with a dual HER1/HER2 inhibitor, lapatinib (GW572016) showed more profound effect on these cell cycle regulators and rapid cell cycle arrest in all the three cell lines tested. Transduction of Tamoxifen treated cells with p-27 peptides (TAT-p27) helped in maintaining quiescence and made the cells resistant to mitogen stimulation (Carroll et al, 2003). These studies evoke the potential of using anti-p27 molecules in future to reverse Tamoxifen resistance. The recent findings that miRNAs also regulate Tamoxifen response in cancer cells is an exciting advance in understanding therapy resistance. Upregulation of miR-221 and/or miR-222 has been directly shown to promote therapy resistance through downregulation of ER α (Zhao et al, 2008).

Recent studies also implicated role of p53 in Tamoxifen mediated cell cycle arrest. Ichikawa et al. reported a concomitant increase in p53 expression and p21, a known CDK2 inhibitor in Tamoxifen treated MCF7 in time and dose dependent manner, suggesting possible role of p53 in mediating the G1 arrest caused by Tamoxifen (Ichikawa et al, 2008).

Future studies, however, are required to understand whether antiestrogens affect the expression of Cyclins at transcriptional level or whether unidentified intermediary players govern this pathway in a similar fashion as p53.

Identifying key G1-S transition regulatory genes that are relieved of pRb mediated repression due to treatment with antiestrogens will be a priority to unravel more downstream players in antiestrogen mediated cell cycle arrest and such studies will further enhance understanding of antiestrogen resistance.

VI. Cell cycle regulators and hormonal therapy resistance

There has been phenomenal advance in our understanding the role of cell cycle regulators in hormonal therapy resistance. Since tamoxifen mediate the cell cycle arrest by deregulating cell cycle regulators, it is perhaps not surprising that aberrant change in cell cycle machinery often contribute to induction of antiestrogen resistance.

We have summarized below the evidence that showed potential role of the regulators of cell cycle machinery in promoting therapy resistance (**Figure 1**).

A. Cyclin D1

Cyclin D1 was originally cloned as an oncogene (Motokura et al, 1991) and over-expression of Cyclin D1 has been noted in over 50% of human breast tumors of all histological types (Gillett et al, 1994; Kenny et al, 1999). There is surmounting evidence to suggest that altered Cyclin D1 expression promotes antiestrogen resistance (Wilcken et al, 1997; Pacilio et al, 1998; Hui et al, 2002). Cyclin D1 binds ER and increases its transcriptional activity (Neuman et al, 1997). This ability of Cyclin D1 to

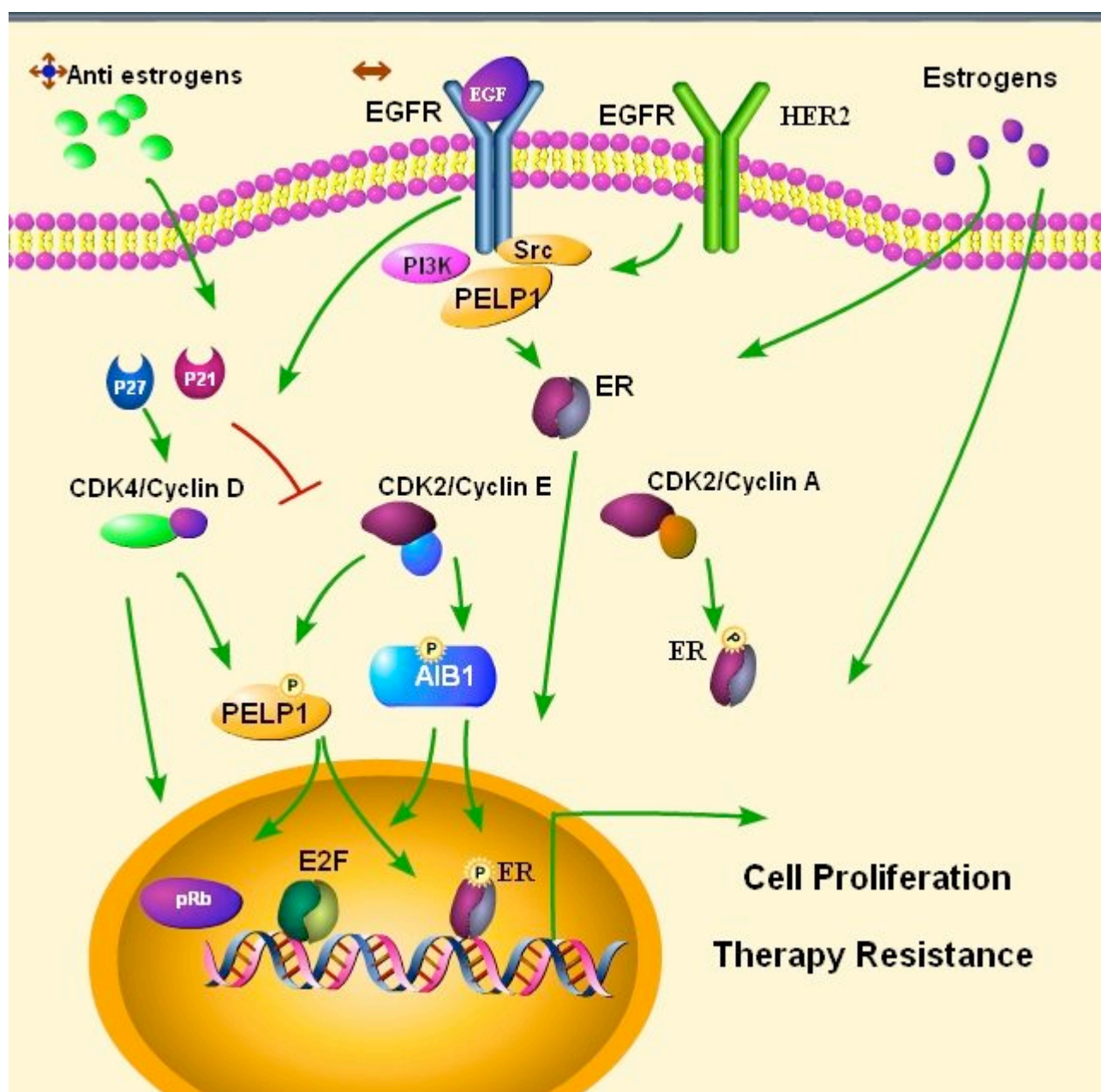


Figure 1. Schematic representation of the current understanding of regulation of hormonal therapy resistance by cell cycle machinery. Convergence of growth factors and estrogen receptor signaling pathways in therapy resistant cells suggest that deregulation cell cycle regulators are likely to contribute to the development of therapy resistance in breast cancer cells.

transactivate ER functions was independent of estrogen stimulation and interestingly, on its CDK4 association as well (Neuman et al, 1997). Over-expression of Cyclin D1 indeed was able to overcome the growth arrest mediated by antiestrogens but Cyclin D1 mutant that is unable to activate CDK4 but having intact ER transactivating potential was not able to promote cell proliferation in the presence of antiestrogens (Bindels et al, 2002). Cyclin D1 is shown to be over-expressed among different Tamoxifen resistant breast cancer cells (Kilker et al, 2004) and Cyclin D1 specific siRNAs restored the sensitivity of these cells to Tamoxifen suggesting therapies targeting Cyclin D1 may have therapeutic effect in hormonal therapy resistant cells (Kilker and Planas-Silva, 2006).

Furthermore, an alternative splice variant of Cyclin D1 named Cyclin D1b is reported to be over expressed in a variety of breast cancers (Betticher et al, 1995; Hosokawa et al, 1997; Wang et al, 2008) and appears to function as a nuclear oncogene (Lu et al, 2003). Cyclin D1b is also known to associate with CDK4 with a weaker kinase activity and over-expression of this alternative transcript Cyclin D1b is shown to overcome the antiestrogen mediated cell cycle arrest (Wang et al, 2008). Unlike Cyclin D1, this effect was independent of ER transactivation as Cyclin D1b lacks nuclear receptor interaction LXXLL motif but retains binding site for CDK4 (Wang et al, 2008).

In addition to activating CDK4, Cyclin D1 is also shown to promote hormonal therapy resistance through other pathways (Ishii et al, 2008). Cyclin D1 is known to mediate STAT3 repression but cells treated with Tamoxifen can potentially reverse this STAT3 repression by the redistribution of Cyclin D1 from STAT3 to ER-complex. This was confirmed by in vivo nude mice assays, where it was shown that growth of Cyclin D1-overexpressing tumors was stimulated by Tamoxifen treatment with concurrent elevation and activation of STAT3 (Ishii et al, 2008). PI3K/AKT or MAPK/ERK1 signaling is also reported to contribute to Cyclin D1 expression and promote to therapy resistance to Tamoxifen underscoring the importance of cross talk between various mitogenic pathways with cell cycle machinery in ultimately achieving antiestrogen resistance (Kilker et al, 2004).

Cyclin D1 negative tumor patients show better relapse free survival upon Tamoxifen-based therapy while Cyclin D1 expression correlated well with poor outcome upon antiestrogen treatment (Rudas et al, 2008). Clinical study with randomized post-menopausal breast cancer patients also show that Cyclin D1 over-expression correlates with poor outcome with Tamoxifen treatment (Stendahl et al, 2004). Similar results were obtained with premenopausal breast cancer patients with Cyclin D1 gene amplification (Jirstrom et al, 2005). Collectively these emerging finding suggest importance of Cyclin D1 as a useful predictive marker in the selection of Tamoxifen-based therapy regime.

B. Cyclin E

Deregulation of Cyclin E in breast cancer model cells has been shown to resist cell cycle arrest mediated by

Tamoxifen and this effect in part was attributed to the aberrant activation of E2F-Rb pathway (Dhillon and Mudryj, 2002). Subsequent studies showed that Cyclin E level showed good correlation with poor relapse-free-survival in patients treated with antiestrogens (Span et al, 2003). Interestingly, Cyclin E was not observed to be good prognostic marker for breast cancer as a whole, however, Cyclin E is a good predictor of antiestrogen resistance (Span et al, 2003; Desmedt et al, 2006). Another important feature of Cyclin E is its tumor specific proteolytic cleavage, yielding low molecular weight (LMW) forms of Cyclin E (Porter et al, 2001). Recent reports suggest that these LMW Cyclin E, lacking varying amount of amino terminal region of whole length Cyclin E, plays a vital role in promoting hormone therapy resistance (Akli et al, 2004). The LMW forms of Cyclin E could complex with CDK2 and accounts for increased CDK2 activity as compared to full length Cyclin E (Akli et al, 2004). LMW-Cyclin E overexpressing MCF-7 cells showed greater resistance toward ICI-182,780 mediated growth arrest as compared to full length Cyclin E and this resistance was attributed to decreased inhibitory effects of p21 and p27 on these LMW-Cyclin E forms (Akli et al, 2004).

C. Cyclin A

Emerging evidences suggest that Cyclin A also play important role in hormone therapy resistance. Detection of Cyclin A over expression by immuno-histochemical methods correlated well with early breast cancer relapse and can be considered a good marker of Tamoxifen resistance (Michalides et al, 2002). Cyclin A is also known to associate with CDK2 and phosphorylates ER and thereby increase its transactivation potential (Trowbridge et al, 1997). Cyclin A/CDK2 complex phosphorylates Ser-104 and Ser-106 located in the AF-1 domain of ER and increase its transcriptional activity (Rogatsky et al, 1999). The ER transactivation through CDK2-Cyclin A phosphorylation is evident in presence and the absence of estrogen stimulation and also with Tamoxifen treatment (Rogatsky et al, 1999). Large scale randomized trials are however required to understand the potential of CDK2-Cyclin A mediated phosphorylation of ER as a prognostic marker for assessing the efficacy of antiestrogen therapy regime.

D. Cyclin dependent kinases

Most downstream events in antiestrogen resistance signaling pathways, like upregulation of various Cyclins ultimately converge upon modulation of Cyclin Dependent Kinases; the most conspicuous of which is the activation of Cyclin Dependent Kinase 2 (CDK2) (Dhillon and Mudryj, 2002; Akli et al, 2004). Apart from CDK2, CDK10 has been recently implicated in hormone resistance. CDK10 is a newly reported player in mediating antiestrogen therapy resistance, identified by functional genomics approach (siRNA screen) (Iorns et al, 2008; Swanton and Downward, 2008). An unbiased loss of function siRNA screen performed by Iorns et al, identified modulators of Tamoxifen sensitivity and found that RNAi mediated downregulation of CDK10 increases ETS2-driven transcription of c-RAF, resulting in MAPK

pathway activation and independence from ER pathway. Loss of CDK10 in ER positive breast cancer was shown to be associated with relapse of cancer after anti-hormone therapy. CDK10 is cdc2 related kinase found to play important role in G2-M progression. While no Cyclins have been identified to associate with CDK10, it is known that ETS2 is interacting partner of CDK10 (Kasten and Giordano, 2001). This low amount of CDK10 in antiestrogen resistant cells were attributed to the methylation of CDK10 promoter in vivo, underscoring the importance of epigenetic changes accompanying the hormone resistance phenotype (Iorns et al, 2008).

E. CDK inhibitors

Down regulation of p21 has been implicated with Tamoxifen resistant phenotype. Somatic deletion of p21 gene in human breast cancer cells demonstrated that these cells were resistant to Tamoxifen mediated growth arrest (Abukhdeir et al, 2008). The mechanism behind this effect was attributed to increased ER phosphorylation at serine 118 by CDK complex upon p21 decrease. Role of ER phosphorylation as an effector of Tamoxifen resistance was elucidated by transfecting p21 null-MCF10A cells with ER cDNA constructs with Serine118 mutated to alanine. These transfected cells became responsive to Tamoxifen, proving that ER activation is the downstream element in p21 mediated Tamoxifen growth resistant phenotype (Abukhdeir et al, 2008). Antiestrogen resistance could be abolished by treating cells with antisense p21 or p27 oligonucleotides, leading to activation of Cyclin Dependent Kinase 2 (Cariou et al, 2000).

Among various molecular pathways implicated in down regulating CDK inhibitors, MAPK/MEK activation is notable (Donovan et al, 2001). MEK inhibitor, U0126 was used to inhibit MEK pathway and re-sensitized to growth arrest by antiestrogen in LY-2 model cells of antiestrogen resistance. Different phospho-isoforms of p27 were detected in these antiestrogen resistant model cells that may contribute toward generating resistance phenotype (Donovan et al, 2001). Detailed studies are however warranted to delineate and correlate specific sites of phosphorylation on p27 with clinical outcome with antiestrogen therapy.

Localization of CDK inhibitors has also been implicated in the development of antiestrogen resistance. Studies have shown that heregulin β 1 over-expression that activates PI3K and MAPK pathway, also promotes p21 localization into cytoplasm (Perez-Tenorio et al, 2006). Tumors with increased cytoplasmic localization of p21 respond poorly with Tamoxifen treatment (Perez-Tenorio et al, 2006). In premenopausal women with early breast cancer, an increase in p27/KIP1 expression was able to predict better relapse free survival upon Tamoxifen combination treatment (Pohl et al, 2003). This trial included 512 randomized patients wherein multivariate analysis revealed decreased p27 expression to be correlated with poor outcome upon combination endocrine therapy. A recent study indicated that p27kip1 is another important target of miR-221 that promotes mediate resistance to hormonal therapy (Miller et al, 2008).

F. Retinoblastoma and E2Fs

Rb-E2F pathway plays a fundamental role in cell proliferation and deregulation is frequently observed in breast cancer. siRNA mediated Rb ablation is able to overcome the growth arrest by antiestrogen treatment and using in vivo xenograft model, Rb deficient tumors were shown to retain the ability to grow in spite of Tamoxifen treatment (Bosco et al, 2007). Furthermore, the same study included analysis of 60 human breast cancer patients treated with Tamoxifen to generate a Rb gene expression signature (Bosco et al, 2007). Another study found that expression of viral T-antigens in breast cancer cells (MCF7) that promote inactivation of endogenous Rb, elicited antiestrogen resistance (Varma and Conrad, 2000). P53 binding ability of T-antigen was however shown not required for this phenotype. In continuation of this work, Conrad and colleagues elucidate the molecular mechanism behind Rb's role in promoting antiestrogen resistance (Varma et al, 2007). Inducible pyLT cell lines were utilized to demonstrate that functional inactivation of pRb can lead to CDK2/Cyclin A activation and reversal of antiestrogen mediated cell cycle arrest. The new hypothesis put forward was that ER⁺ Rb⁻ tumors showing increased CDK2 activity and resulting hormone therapy resistance can be targeted by agents blocking CDK2. Currently many such CDK2 targeting drugs (although not very specific ones) are available in clinical trials and need to be evaluated in the context.

G. c-Myc

c-Myc is a well known cell cycle regulator and oncogene frequently up regulated in breast cancer. It is also one of the earliest estrogen responsive gene, showing a noticeable increase in protein level within 15 min of estrogen treatment (Dubik et al, 1987). C-myc expression when induced in MCF-7 using Tet-on expression system could potentially abrogate antiestrogen mediated growth arrest (Venditti et al, 2002). Similar results were obtained in a different study, wherein over expression of c-myc down regulated p21 expression and mediated antiestrogen resistance (Mukherjee and Conrad, 2005). C-myc expression can rescue the G1 arrest mediated by Tamoxifen by activating CDK2/Cyclin E complex and further phosphorylation of p130 (Prall et al, 1998a). Involvement of c-myc in regulating p21 expression levels and contributing to emergence of antiestrogen resistance is also reported (Mukherjee and Conrad, 2005). p21 levels in antiestrogen resistant cells increased when treated with c-myc siRNAs, suggesting important role of c-myc in downregulating p21 levels and promoting hormonal therapy resistance (Mukherjee and Conrad, 2005).

From the above mentioned studies, we present an interesting case that cell cycle regulators play a vital role in the emergence of hormone therapy resistance. However, the studies performed so far do not provide clear distinction of using cell cycle regulators as prognostic markers of therapy resistance or therapeutic targets against resistant cells. The key challenge in this area is to unequivocally show that targeting cell cycle regulators can potentially reverse the hormone therapy resistance but the side effects may limit their use as evidenced by recent

studies. Targeting kinase functions of CDK2 is a feasible option and currently there are some ongoing clinical studies employing pan CDK inhibitors against non-small cell lung cancer like r-roscovitine (Seliciclib or CYC202). Our lab has recently tested the efficacy of combinatorial usage of r-roscovitine with Tamoxifen against various hormone resistant cell lines like MCF-tam (resistant to Tamoxifen), MCF-7-Her2 (overexpressing Her2), and MCF7-PELP1 (overexpressing PELP1) and found encouraging results in sensitizing these cells to Tamoxifen treatment (Chandrasekharan Nair et al, 2008b). Another possibility to overcome toxic side effects would be to explore nanotechnology methods that allow cancer cell specific delivery of the cell cycle inhibitors reducing toxic side effects. Such combinatorial use of cell cycle inhibitors along with classical hormone therapy represents a novel therapeutic modality to circumvent the problem of toxicity and to enhance therapeutic success.

VII. Conclusions and Future Direction

The estrogen receptor (ER) plays a central role in the progression of breast cancer and endocrine therapy is widely used to target ER+ve breast cancer. Despite the positive effects, de novo and/or acquired resistance to endocrine therapies frequently occur. Most downstream events in the resistance signaling pathways appear to converge upon modulation of cell cycle regulatory proteins. Evolving evidence suggests that cell cycle machinery cross talk with estrogen receptors, ER-coregulators and growth factor receptors and such interaction play a role in the development of therapy resistance. It is therefore of great interest to understand how cell cycle machinery promotes therapy resistance. Since cell cycle dependent kinases cross talk with nuclear receptors and coregulators to regulate various downstream genes, we believe that associated nucleosomal histone modification via methylation and acetylation could play a vital role in therapy resistance. There is scarcity of studies toward understanding cell cycle dependent histone/DNA modifications and epigenetic changes that contribute toward acquiring hormone therapy resistance. Similarly, identifying newer substrates of CDKs and investigating their potential role in therapy resistance will provide novel insights into the mechanistic basis of Tamoxifen resistance. Combinatorial therapy using CDK inhibitors along with conventional hormone therapy is a feasible option to resensitize the cells against hormone therapy resistance. Future microRNA profiling studies is expected to identify new miRNAs that regulate cell cycle machinery, thus increase the repertoire of novel targets for interfering hormone therapy resistance. Future studies are also warranted in safe delivery of cell cycle inhibitors utilizing new technologies (such as targeted nano particles) to enable to use these new drugs with less side effects. We strongly believe that further understanding of the molecular mechanisms by which tumor cells use cell cycle machinery to acquire therapy resistance will provide novel therapeutic targets, which in conjunction with conventional hormone therapy will be useful in targeting therapy resistant tumors.

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2. **Nair BC** and Vadlamudi RK. ZD6474 coerces breast cancer for an apoptotic journey. *Cancer Biology and Therapy*. 9:8, 1-3; 2010

ZD6474 coerces breast cancer for an apoptotic journey

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Cancer is a complex disease with a plethora of incriminated proteins at the helm, all contributing to the successful survival of cancerous cells. Breast cancer is the second leading cause of death and holds the dubious distinction of being fatal, owing to serious shortcomings in diagnosis and lack of effective treatment modalities. Approximately 192,370 new cases of invasive breast cancer were expected to occur among women in the US during 2009.¹ With the growing repertoire of oncogenic proteins involved in breast cancer, the list of potential drug targets has also increased steadily over the last decade. One of the expected consequences of having a multitude of molecular targets is the debate over developing a 'single wonder pill' affecting different drug targets or alternatively using cocktail preparations of drugs known to affect diverse molecular pathways to harness combinatorial benefits.

Two important pathways are quintessential for the cancer growth: (1) the hypersensitive mitogenic pathway, which promotes increased and aberrant cell proliferation and (2) the angiogenic pathway, which contributes to neo-vascularization, a process of sprouting new blood vessels to meet the nutrient requirements of cancerous tissue. Cancer cells can acquire the capacity for autonomous and dysregulated proliferation through the uncontrolled production of growth factors or through abnormal, enhanced expression of growth factor receptors.² Aberrant expression and regulation of epidermal growth factor receptor (EGFR) pathway has been found in many different solid tumor types including aggressive metastatic breast cancer³ and hormone therapy resistant tumors.⁴ The EGFR is a member of the ErbB family

of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), ErbB-3 and ErbB-4.^{5,6} Ligand binding to the EGF receptor induces formation of homo- and heterodimers with other ErbB members, leading to activation of the intrinsic kinase via autophosphorylation, which stimulates various intracellular signaling cascades, leading to mitogenesis.⁷ In addition, bidirectional cross-talk between EGFR and the estrogen receptor contributes to reproductive organ physiology and pathophysiology.⁸ Current anti-EGFR therapies focus on using either monoclonal antibodies against EGFR (e.g., cetuximab and panitumumab)⁹ or using drugs that block receptor kinase function (e.g., lapatinib, gefitinib and erlotinib);¹⁰ many of these anti-EGFR drugs are currently in breast cancer clinical trials.

Tumor angiogenesis, on the other hand, is mostly regulated by vascular endothelial growth factor (VEGF), an endothelial-specific mitogen, and its associated tyrosine kinase receptor (VEGFR). There are two types of angiogenic VEGFR: flt-1 (VEGFR-1) and flk-1 (VEGFR-2). In most of the cancers, VEGFR-2 is the predominant type.¹¹ Overexpression of VEGFR and associated pathologies are found in both early and metastatic breast cancer,¹² making it a compelling drug target. Targeting strategies for metastatic breast cancer include either using monoclonal antibodies that specifically block VEGF from binding its receptor or using drugs that inhibit VEGFR tyrosine kinase activity.¹³

Mitogenic and angiogenic pathways work distinctly with different biological endpoints but often cross-talk through a

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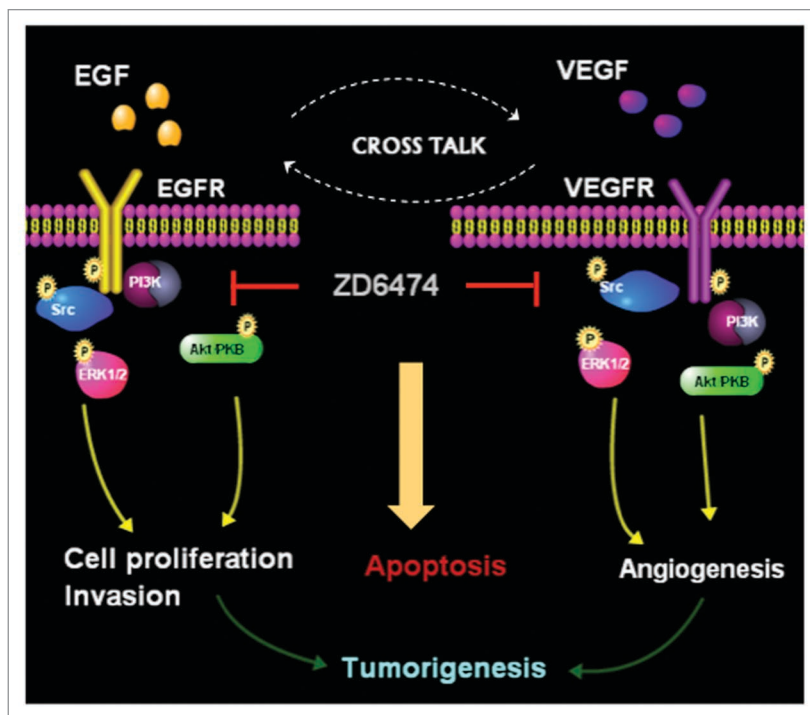


Figure 1. Schematic representation of the EGFR and VEGFR signaling cross talk that contributes to tumor cell proliferation and angiogenesis. Dual inhibition of EGFR and VEGFR signaling by ZD6474 drives cells towards apoptosis.

series of autocrine and paracrine events, which uniquely presents a better therapeutic option of including a combinatorial use of anti-EGFR and anti-VEGFR therapies (Fig. 1). While EGF treatment has been shown to upregulate VEGF-mediated angiogenesis in many cancers, aberrant activation of the VEGF pathway is also responsible for the emerging anti-EGFR therapy resistance.¹⁴ The situation wherein a single multi-targeting drug could block both these pathways could be tremendously beneficial to patients by causing double jeopardy in cancer cells. In this issue of *Cancer Biology & Therapy*, Mandal and colleagues have shown that ZD6474, an oral anilinoquinazoline drug not only inhibits EGFR- and VEGFR-mediated signaling cascades in breast cancer cell lines but also induces apoptosis.¹⁵ ZD6474 binds to the intracellular kinase domain of the receptor tyrosine kinase and inhibits downstream signaling events.¹⁶ The authors report that ZD6474 induces cell cycle arrest in the G₀/G₁ phase and suppresses cell proliferation in a wide variety of breast cancer cell lines irrespective of their estrogen receptor status. Interestingly, apoptosis occurred in more than 50% of

all ZD6474-treated cells as corroborated by a DNA ladder formation assay, PARP cleavage, upregulation of the pro-apoptotic gene, Bax, and downregulation of Bcl-2. As expected from a dual kinase inhibitor, ZD6474 treatment reduced levels of phospho-EGFR and phospho-VEGFR indicating inactivation of EGFR and VEGFR and further blocking both MAPK and PI3K pathways. EGF-mediated phosphorylation of MAPK and phosphorylation of AKT were both reduced in breast cancer cell lines upon treatment with ZD6474. The finding that ZD6474 can act as both cytostatic and cytotoxic agent and that induction of apoptosis at an IC₅₀ dose occurs in a time-dependent manner is quite interesting and no doubt, a potential criterion for determining the appropriate dosage if the drug is approved for human breast cancer clinical trials.

Using a soft colony agar assay, the authors showed that ZD6474 has the potential to reduce EGF-mediated colony formation. ZD6474 also drastically reduced invasion and migration of all breast cancer cells when tested by a Boyden chamber assay. In addition to these in vitro experiments, the anti-proliferative

action of ZD6474 was also demonstrated using an in vivo xenograft transplantation assay. Compared to the untreated control group, the ZD6474-treated group had significantly smaller tumor sizes and tumor weights. Immunohistochemical analysis of tumors not only revealed less ki-67 staining but also revealed less phospho-EGFR and phospho-VEGFR status in the drug-treated group. In addition, tumor tissues from the treated group were positive for TUNEL staining, corroborating the in vitro findings that ZD6474 induces apoptosis in breast cancer cell lines.

In spite of ZD6474's impressive effect on breast cancer, it must be noted that the molecular pathways affected by ZD6474 may not be breast cancer specific. ZD6474 has also been shown to induce cell cycle arrest and apoptosis in human nasopharyngeal carcinoma,¹⁷ suggesting that ZD6474 has the potential to act on variety of cancers and is a promising candidate for clinical trials against various cancers. The dire need for drugs against breast cancer, however, overrides this issue. Since a recent study found that EGFR expression is more common in breast tumors in younger and black women as well as an EGFR association with lower hormone receptor levels,¹⁸ ZD6474 may improve outcome of these groups of patients. It is worth studying how ZD6474 performs on tamoxifen and letrozole resistant breast cancer cells, which differ from the hormone-sensitive tumors through acquisition of various deregulations in both EGFR and VEGFR pathways. Another question that lingers is how much more effective are these dual kinase inhibitors than single EGFR- or VEGFR-targeting drugs. A comparative study should be envisaged to address whether the blockade of cross-talk between EGFR and VEGFR is the primary reason for the enhanced anti-tumor activities of ZD6474. ZD6474 has indeed brought new excitement to the field of breast cancer drug discovery and future studies are very likely to open up new avenues to formulate treatment modalities against different types of breast cancer.

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Endocrine Therapy and Resistance

Modulation of hormone therapy resistance by CDK2-PELP1 axis.

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Abstract

Abstract #3022

Background: The estrogen receptor (ER) plays a central role in the progression of breast cancer. Current endocrine therapy for ER+ve breast cancer involves modulating ER-pathway using Tamoxifen, and blocking peripheral estrogen (E2) synthesis by Aromatase inhibitors. Despite the positive effects, *de novo* and/or acquired resistance to endocrine therapies frequently occur. Although mechanisms for hormonal therapy resistance remains elusive, *most downstream events in these pathways converge upon modulation of cell cycle regulatory proteins including upregulation of Cyclin E and A, along with activation of Cyclin Dependent Kinase 2 (CDK2)*. ER signaling complexes are known to recruit various co-regulatory proteins and recent evidences suggest that deregulated expression, localization and activity of ER coregulators also plays vital role in hormonal resistance. In this study, we found that CDK signaling regulates ER coregulator PELP1 function via phosphorylation leading to hormonal resistance.

Methods: Significance of CDK2 axis in the therapy resistance was tested using breast cancer models cells that acquired resistance to endocrine therapy and by using chemical inhibitors that block CDK2 activity. Immunoprecipitation, and confocal analysis was used to confirm protein-protein interactions. *In*

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vitro kinase and ortho-phosphate labeling assays were used to test CDK2 phosphorylation of PELP1. Utilizing Breast cancer model cells that express PELP1 mutants that cannot be phosphorylated by CDK2, we examined the significance of PELP1 phosphorylation in cell cycle progression. Using PELP1 siRNA nanoparticles, we tested the effect of PELP1 knockdown in hormone therapy resistance.

Results: ER coregulator PELP1 interacts with CDK2 upon E2 stimulation. *In vitro* kinase assays using both purified CDK2/CyclinE and CDK2/CyclinA complexes showed that full length PELP1 is a potential substrate of CDK2. PELP1 exhibited phosphorylation at the time points that corresponds to CDK2 activation in MCF7 cells. PELP1 overexpression increases E2F luciferase activity while PELP1 mutants that lack CDK2 sites failed to enhance the E2F activity. CDK2 mediated phosphorylation of PELP1 is important for PELP1 regulation of E2F and ER target genes. Combination therapies using PELP1 siRNA nano particles or Roscovitine along with tamoxifen or letrozole, sensitized the therapy resistance cells for endocrine therapy.

Conclusions: We have identified ER coregulator PELP1 as a novel substrate of CDK2. Because CDK2 activity is deregulated in breast tumors and implicated in therapy resistance, our findings suggests that CDK2-PELP1 axis deregulation may contribute therapy resistance. Combinatorial therapeutic strategy using Roscovitine along with PELP1 siRNA nanoparticles will provide new therapeutic opportunity to increase the sensitivity of hormone resistant cells to Tamoxifen and Letrozole therapy. These studies were supported by DOD breast cancer grant BC083207.

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